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Expression of Glucose Isomerase Gene from *Bacillus licheniformis* in *Escherichia coli.*

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Bacillus licheniformis 포도당 이성화 효소 유전자의 *Escherichia coli*에 발현

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A *Bacillus licheniformis* ATCC31667 gene coding for a glucose isomerase has been cloned and expressed in glucose isomerase negative mutant of *Escherichia coli.* A recombinant plasmid, constructed by ligation of a EcoRI fragment of *B.licheniformis* chromosomal DNA to vector plasmid pBR322, was expressed glucose isomerase positive in *E.coli* LE392-6 with growth on minimal medium containing xylose as a sole carbon source. This recombinant plasmid, designated pBGI6, had the insert of 4.1Kb of *Bacillus* gene in EcoRI site, and restriction map of the plasmid was established. The plasmid pBGI6 was very stable after 10days of serial transfer to a fresh medium. The activity of glucose isomerase from the transformed cell containing pBGI6 was increased about 20 fold than its wild type of host.

Glucose isomerase (E.C.5.3.1.5.) which catalyzes the reverse isomerization of glucose to fructose was firstly reported by Marshall and Kooi in 1957. The use of glucose isomerase (GI) for the production of high fructose syrup has assumed considerable importance in the food industries. Fructose has been recognized as a good alternate source of sugar due to its relatively high sweetness and other desirable physico-chemical properties. GI was produced in *Streptomyces* sp., *Bacillus* sp., *Aerobactor* sp., *Lactobacillus* sp., *Actinomycetes* sp., *Pseudomonas* sp., and *E. coli* and many other microbial sources on this enzyme were reported. (Takasaki *et al,* 1969; Boguslawski et. al., 1982; Tsumura and Sato 1965; Yamanaka 1963; Natake et al., 1963; Natake and Yoshimura 1964; Vaheri et al., 1977).

Almost every known GI is an intracellular microbial enzyme and also catalyzes isomerization of xylose to xylulose which is very important in utilization of xylan, a polymer of xylose (Yamanaka 1957; Isabel and Roncero 1983; Parkand et al, 1974; Mutze et al, 1983; Dellweg et al., 1984; Jeffries 1981). Recently, there are several reports on GI gene cloning for the enhancement of GI activity and also on the regulatory system in *E. coli* by gene amplification. (Wovcha et al, 1983; Schellenberg et al, 1983; David et al, 1970; Shamanna et al. 1979). Previously, we also reported on GI cloning system in *Streptomyces.* (Kho 1984)

This report describes the construction and selection of a recombinant plasmid coding for GI and expression of *Bacillus* gene in *E. coli.* We also describe the production of the GI and its activity in the cloned cell.

MATERIALS AND METHODS

Bacterial strains, plasmid and media.

The donor strain, *B. licheniformis* ATCC 31667, was chosen for its high GI activity and commercial properties (Boguslawski, 1982). The recipient strains used in the cloning were *E. coli* LE 392-6 (glucose isomerase negative mutant drived from *E.coli* LE 392) and *E. coli* JC1553 (Wovcha, 1983) obtained from University of Wisconsin. These strains cannot use D-xylose as a sole source of carbon and energy. The cells were grown at 37 °C with agitation in LB; tryptone 1%, yeast extract 0.5%, NaCl 0.5%, pH 7.0-7.5. Cloned cells of *E. coli* LE 392-6 were grown in standard M9 medium containing tryptophan 20mg/l, methionine 50mg/l, thiamine 10mg/l, leucine 40mg/l, proline 5mg/l, threonine 35mg/l, and xylose 1% (MM1). Transformants of *E. coli* JC 1553 were selected on the minimal salts medium of Vogel-Bonner supplemented with L-arginine, leucine, histidine, methionine (each at 40mg/l), and 1% xylose as a sole carbon source (MM2). The plasmid pBR322 was purchased from BRL Inc (Bethesda, M.D. U.S.A.). When required, antibiotics (Sigma) were added to the medium at a final concentration (μg/ml) of 10 and 30 for tetracycline and ampicillin, respectively. The solid agar medium was contained 1.5% agar (Difco).

Chemicals and reagents.

Tris, E.D.T.A., antibiotics, N.T.G., amino acids, lysozyme and RNase were obtained from Sigma. Agarose type I and V for electrophoresis gel were also from Sigma. The restriction enzymes; EcoR I, Bam HI, Pst I, Hind III, Sal I, Pvu II, XhoI, BglII, Sst I and Bcl I were purchased from BRL Inc.(Bethesda, M.D., U.S.A.). T_4 DNA ligase was also obtained from BRL.

Isolation of XYL$^-$ mutant.

Glucose isomerase negative (GI$^-$) mutant was drived from *E. coli* LE 392 using NTG. The mutagenized culture of *E. coli* LE 392 was spread MM1 supplemented with 1% glucose and replicated to MM1 supplemented with 1% xylose and xylulose. GI$^+$ cells can grow on xylose minimal medium but GI$^-$ mutants can not. Therefore, the colony was screened as a GI$^-$ mutant which did not grow on xylose but grew on xylulose or glucose as a sole carbon source.

Gel electrophoresis.

DNA was analyzed by electrophoresis (horizontal type) on 1% of agarose gel using 0.89M Tris-borate buffer (pH 8.0) contained 5μg/ml of ethidium bromide. Gels were run at 75volt for 3hrs and photographed with U.V. transilluminator (U.V. product. Inc) using polaroid film (8.3x 10.8cm, type 667). Restriction fragments of Hind III-digested λ DNA were used as size standards.

DNA preparation

Chromosomal DNA was prepared from *Bacillus licheniformis* ATCC31667 log phase cultured aerobically as described by Marmur (1961). Extraction of plasmid DNA from recombinant clone was performed according to the microscale plasmid DNA extraction described by Holmes and Quigley. (1981).

Restriction endonuclease digestion and DNA ligation

Bacillus licheniformis DNA was partially digested with restriction endonuclease under the conditions described by the suppliers, respectively. This digestion gave fragments between 1 and 20 kb in size. After digestion, DNA was phenol-chloroform extracted and ethanol-precipitated.

Ligation reaction was carried out in 6.6mM $MgCl_2$, 66mM Tris (pH7.6), 1mM ATP, 10mM Dithiothreitol using 2μg of same restriction endonuclease digested plasmid pBR 322, 4μg of digested *B. licheniformis* chromosomal DNA and 1 unit of T_4 DNA ligase in a final volume of 30μl. The reaction mixture was incubated at 10 °C for more than 16hrs.

Transformation

Transformation of *E. coli* was performed according to prescribed method (Cohen et al., 1972). The overnight cultured cells in LB broth were transferred to fresh LB broth and more cultured at 37 °C with shaking to A_{590} = 0.3 - 0.6. The cells harvested and resuspended in 1/2 volume of culture medium with 50mM $CaCl_2$ and incubated at 0 °C for 1hr. These cells pretreated with $CaCl_2$

were resuspended in 1/20 volume of original broth with 50mM $CaCl_2$ and incubated at 0 °C for more than 1hr. And then added 10 μl of ligased DNA (0.3μg to 1μg of total DNA) to 0.2ml of competent cell. After incubation at 0°C for 30min., temperature was elevated to 42 °C for 2 to 3min. for uptake of DNA to *E. coli.* These heat shocked cells were diluted to 1ml with LB broth and incubated at 37 °C for 1.5 to 3 hrs to allow for expression before plating. The transformed cells were plated on MM1 medium containing xylose and antibiotics. If the GI gene was cloned, the transformants would be grown on xylose minimal medium and resistant to ampicillin or tetracycline originated from plasmid pBR 322, while their host would not. These results indicate that the cells contain foreign gene with insertion of DNA fragment that complement the GI gene from *B. licheniformis.* In order to eliminate the possibility of gene reversion in the cloned cell, the DNA was extracted from the genetically complemented colonies and its restriction pattern was analyzed.

Enzyme Assay

The GI activity was assayed by measuring the amounts of fructose which was conversed from glucose by GI (Yamanaka et al., 1957; Kasumi et al., 1982). The reaction mixture was contained 1ml substrate solution (1 M glucose, 20mM potassium phosphate buffer pH 7.2, 2.5mM $CoCl_2$) and equal volume of cell suspension. The bacterial cells were grown in 20ml of production medium at 37 °C with shaking for 24hrs, harvested (8,000 rpm, 4 °C, 10min.), washed with substrate buffer (20mM potassium phosphate pH 7.2, 2.5mM $CoCl_2$) and suspended in 2ml of the same buffer. The reaction mixture was incubated at 60 °C for 1hr. and stopped by the addition of 2ml of 0.5M perchloric acid.

D-fructose produced was determined by the resorcinol method (Ashwell, 1957) at the final concentration of below 100μg per ml. One unit of the GI activity was defined as the produced one micromole of fructose under the conditions shown above.

Table 1. *Growth of E. coli strains on MM 1 agar containing glucose, xylulose and xylose as a sole carbon source.*

Strain	glucose	xylulose	xylose
E. coli LE392	+++	+++	+++
E. coli LE392−4	+++	—	—
E. coli LE392−6	+++	+++	—
E. coli JC1553	+++	+++	—

+++ : Well growth
— : No growth

Table 2. *Growth of E. coli LE392 and LE392−6 on a sole carbon source of xylose or xylulose.*

Incubation period (day)	Xylose		Xylulose	
	E. coli LE392	*E. coli* LE392-6	*E. coli* LE392	*E. coli* LE392-6
1	+	—	+	+
2	++	—	++	++
3	+++	—	+++	+++
4	+++	—	+++	+++
5	+++	+	+++	+++

+++ Well growth
\+ Growth
— No growth

RESULT AND DISCUSSION

Isolation of xylose negative mutants

GI^- mutant strain, *E. coli* LE392-6 was chosen with NTG treatment as the recipient in a tranformation experiment. This mutant showed poor growth on MM1 agar medium containing 1.0% D-xylose as a sole carbon source. However the organism was shown very good growth on MM1 agar medium supplemented with D-glucose or D-xylulose (Table 1). *E coli* LE-392-6 was very stable in its phenotype during the incubation (Table 2). These results showed that the GI^- mutant could not utilize xylose due to deletion of XYL gene.

Cloning of the glucose isomerase gene from *B. licheniformis* to *E. coli.*

The chromosomal DNA from *B. licheniformis* was isolated and partially digested with Pstl, Hind

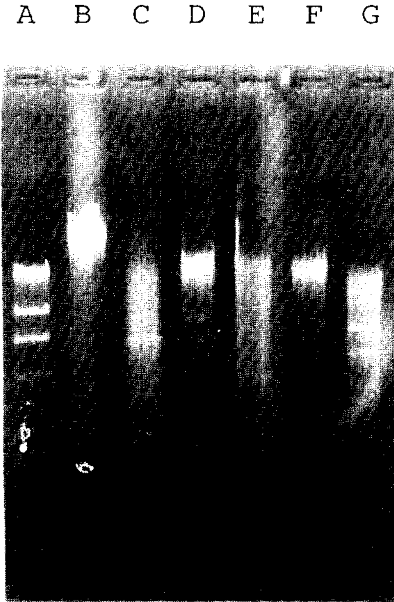

Fig. 1. *Agarose gel eletrophoresis of Bacillus licheniformis ATCC 31667 chromosomal DNA digested by various restriction enzymes.*
Lane A: λ DNA were digested with Hind III as a size marker.
B: Non digested *B. licheniformis* chromosomal DNA.
C: EcoR I, D: BamH I, E: Pst I, F: Hind III,
G: Sal I.

Fig. 2. *Identification of plasmid DNA by agarose gel electrophoresis.*
Lane A: Hind III-digested λ DNA as size marker.
B: Chromosomal DNA of *E. coli* LE392-6 (host).
C: pBR 322, D: pBGI 6.

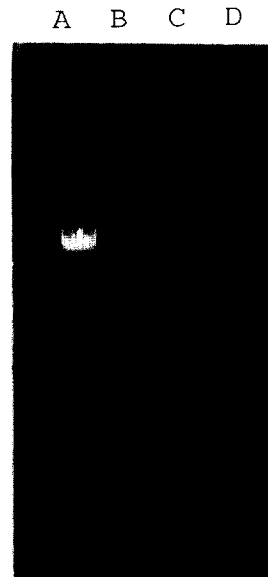

Fig. 3. *Electrophoresis of plasmid pBGI 6 DNA digested by EcoR I.*
Lane A: Hind III-digested λ DNA as marker.
B: pBR322 digested by EcoRI
C: pBGI6 digested by EcoRI
D: Chromosomal DNA of *E. coli* LE392-6

III, Sal I, BamHI, or EcoRI (Fig. 1).
Fragments ranging from 1 to 20kb were generated for the cloning experiment. To increase the yield of recombinants, a 3-4 molar excess of partially digested chromosomal DNA was used during the ligation step. Recombinant DNA from ligation mixture was transformed into *E. coli* LE 392-6. The transformed cells were spread on MMI agar plate supplemented with xylose after 3 days of shaking incubation in MM1-xylose broth at 37 °C. Transformants were selected by their ability to grow on MM 1 agar containing xylose and antibiotics (ampicillin, tetracycline). Two types of transformants from the EcoRI genomic library were obtained and a large colony grown faster was selected. The clone was purified on selective media by single colony isolation technique prior to plasmid DNA extraction. The recombinant plasmid was extracted from the cloned cell (Fig.

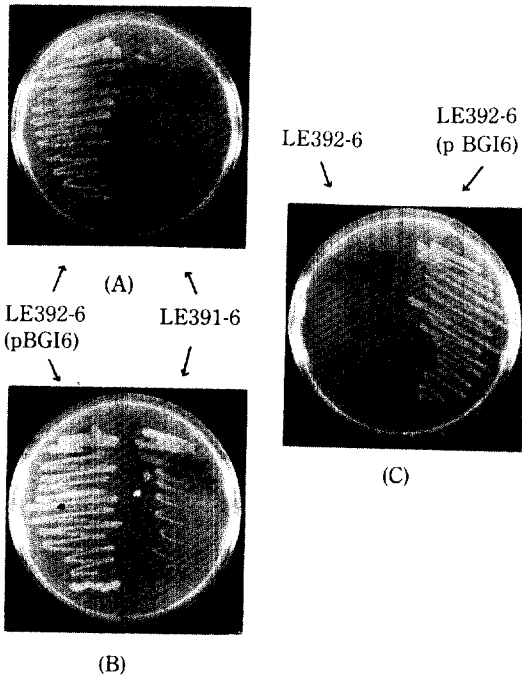

Fig. 4. *Growth of E. coli LE392-6 (pBG16) and Le392-6 on xylose minimal agar incubted at 37°C for 1 day (A), 5 days (B) and xylose minimal agar containing tetracycline incubated 37°C for 5 days (C).*

2), named pBGI 6, and retransformed to *E. coli* LE392-6, 100% of the retransformants were shown excellent growth on MM1 agar containing xylose and antibiotics, but their host was not (data not shown). The plasmid pBGI 6 was 8.5 kb in total length with an insert of 4.1 kb fragement determined by observing its electrophoretic mobility on agarose gel relative to that of Hind III-cut λDNA as molecular marker (Fig. 3).

Stability of pBGI6 and Retransformation

The stability of pBGI 6 was checked with utilization of xylose and antibiotic resistance. The single cloned cell was grown in fresh complete medium and spread on MM1 containing xylose and antibiotic agar plate. After 10 times of serial transfer, the cloned cell was shown good growth on xylose minimal medium and resistance against antibiotics (Fig. 4).

The recombinant plasmid pBGI6 was very stable (Table 3) and also agreed with gel pattern (Fig. 5). The pBGI6 was transformed to another

Table 3. *Stability of pBGI6 by growth on minimal agar plate containing xylose and tetracycline.*

Number* of transfer	*E. coli* LE392-6 (pBGI 6)**		*E. coli* LE392-6***	
	Xylose	Xylose+ Tetracycline	Xylose	Xylose+ Tetrcycline
1	+++	+++	-	-
2	+++	+++	+	-
3	+++	+++	+	-
4	+++	+++	+	
5	+++	+++	+	-
6	+++	+++	+	-
7	+++	+++	+	
8	+++	+++	-	-
9	+++	+++	-	
10	+++	+++	+	-

— no growth.
\+ growth
++ + Well growth

* Full grown cells in LB broth were transferred every day in fresh LB broth
** Incubate for 2 days at 37℃ after inoculation.
*** Incubate for 5 days at 37℃ after inoculation.

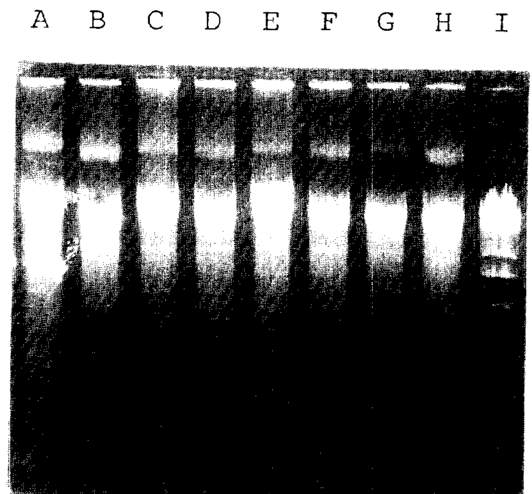

Fig. 5. *Electrophoresis of pBGI6*
Lane A-H: 3-10 days grown in LB broth.
I: Hind III digested λ DNA as size marker.

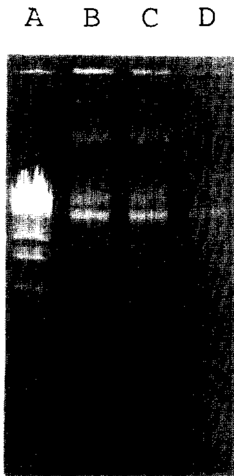

Fig. 6. *Electrophoresis of plasmid pBGI6 from transformed E. coli JC 1553.*
Lane A: Hind III digested λ DNA as size marker.
B,C: plasmid pBGI6
D: Chromosomal DNA of *E. coli* JC 1553.

GI⁻ mutant strain, *E. coli* JC 1553. All transformants were shown well growth on xylose minimal agar plate and their restriction patterns were same as those of *E. coli* LE 392-6 (pBGI6) (Fig. 6). From these results, we can conclude that the recombinant plasmid, pBGI6, surely code for glucose

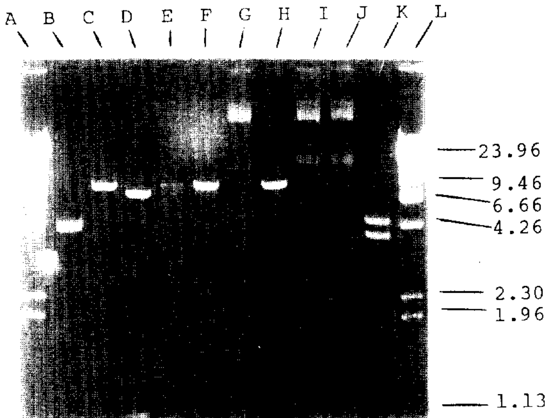

Fig. 7. *Electrophoresis of plasmid pBGI6 DNA digested by various restriction enzymes.*
Lane A,L: Hind III digested λ DNA as size marker.
B: EcoR I, C:Sal I, D:Hind III, E:BamH I, F:Pst I, G:Xho I, H:Bgl II, I:Sst I, J:Bcl I, K:pvu II.

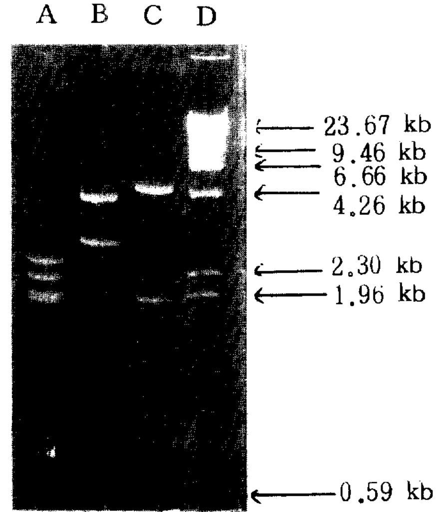

Fig. 8. *Electrophoresis of double digested plasmid pBGI6 DNA*
Lame A: Pvu II and EcoR I, B:Hind III and EcoR I
C: Hind III and PvuII
D: Hind III digested λ DNA as size marker.

isomerase and very stable.

Characterization of the recombinant plasmid pBGI6.

In order to establish a restriction map of pBGI6, plasmid pBGI6 was digested with EcoRI, Hind III, BamH I, Pst I, Xho I, Bgl II, Sst I, Bcl I and Pvu II (Fig. 7). There are no restriction site for XhoI, SstI or BclI and have one cleavage site for SalI, Pst I, BamHI and BglII, probably, originated from pBR322. Two sites for EcoRI, Hind III and Pvu II were obtained. Double restriction enzyme digestion of the plasmid were shown in Fig. 8. Four fragments were obtained from each double digestion; 1.7kb, 2.07kb, 2.3kb, 2.4kb by EcoRI and Pvu II; 0.03kb, 1.1kb, 3.0kb, 4.3kb by by EcoRI and Hind III, 10.6kb, 7.6kb, 1.13kb, 14.7kb by Hind III and Pvu II. From these gel patterns, the restriction map was established (Fig. 9).

Expression of recombinant plasmid pBGI6.

The expression of pBGI6 was investigated by measuring GI activity compared the transformant with its host and donor strain. Cultures of *B.licheniformis* ATCC 31667 (donor), *E. coli* LE

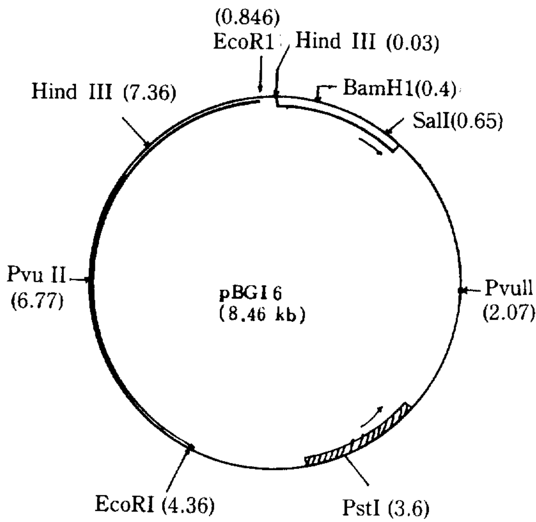

Fig. 9. *Restriction map of plasmid pBGI6*
Single line: pBR 322
Double line: Insert. 4.1 kb of *B. licheniformis* gene
(open bar): Tetracycline resistant region
(hatched bar): Ampicilline resistant region

392-6 (host) and LE392-6 (pBGI6) were grown aerobically overnight at 37 °C on LB broth containing tetracycline (10μg/ml) and portions of each (1%:V/V) were used to inoculate fresh flask of 20ml of production medium (yeast extract 0.25%, peptone 1%, NaCl 0.5%, $MgSO_4$ $7H_2O$ 0.05%, xylose 1%). After growth for 24hrs, the cells were collected by centrifugation (Hitachi, 8,000 rpm, 4 °C 10 min), washed and resuspended in 20 mM potassium phosphate buffer (pH7.2).

This cell suspension were used for assay of GI activity. GI activity from the cloned strain *E. coli* LE 392-6 bearing pBGI6 was about 20 fold increase than its host *E. coli* LE 392-6 but appeared 90% of GI activity of its donor strain *B. licheniformis* (Table 4). It is revealed that the increasing of GI activity in *E. coli* was caused by recombinant plasmid pBGI6 and GI system in *E. coli* is different from *B. licheniformis*. (Isabel et al. 1983; Lam et al. 1970; Handerson et al. 1977). The GI activity

Table 4. *D-Glucose isomerase activity* of E. coli LE 392-6, LE 392-6(pBGI 6) and B. licheniformis ATCC 31667*

Strains	Glucose Isomerase Activity (μmole/ml)
B. licheniformis ATCC 31667	60
E. coli LE 392-6	2.2
E. coli LE 392 (pBGI 6)	50

* D-Glucose isomerase assays were carried out at 60℃ with 20mM potassium phosphate (pH 7.2), 2.5mM $CoCl_2$, 1 M glucose and cell mass in a volume of 1 ml. The accumulation of fructose were measured by the Resorcinol method.

Table 5. *Effect of xylose and glucose on glucose isomerase activity**

Addition	Strains	G. I. activity (μmole/ml)
No	*E. coli* LE 392-6	4
	E. coli LE 392-6 (pBGI6)	50
Xylose (1 %)	*E. coli* LE 392-6	5
	E. coli LE 392-6 (pBGI6)	80
Glucose (1 %)	*E. coli* LE 392-6	2
	E. coli LE 392-6 (pBGI6)	30

* Assay conditions are described in Table 4.

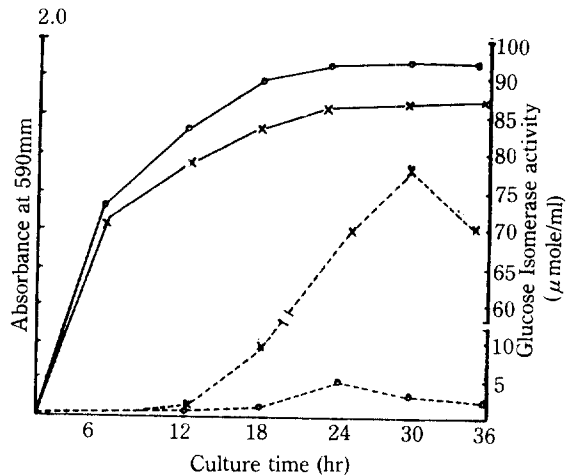

Fig. 10. *Growth and glucose isomerase activty of E. coli LE 392-6 and LE 392-6(pBGI6)*
—— ○ —— Growth of LE392-6
—— × —— Growth of LE 392-6 (pBGI6)
········ ○ ······ Activity of GI of LE 392-6
········ × ······ Activity of GI of LE 392-6 (pBGI6)

was induced by xylose addition to the broth and decreased by presence of glucose. (Table 5) The relativity of cell growith and productivity of GI were also determined as shown in Fig. 10. The maximum GI activity was obtained after 24 to 30 hrs of cultivation under optimum conditions.

적 요

포도당 이성화효소를 coding하는 *Bacillus licheniformis* ATCC31667의 유전자를 *Escherichia coli* LE 392-6에 클로닝하였다. *Bacillus licheniformis* 염색체 DNA를 분리하고 제한효소인 Pst I, HindIII, Sal I, EcoR I, BamH I으로 절단한 후 운반체 plasmid인 pBR322에 연결하고 포도당 이성화 효소 negative인 *E. coli* LE 3926-6에 형질전환하였다. 이중 EcoRI 제한효소를 사용한 것만이 glucose isomerase positive로 전환되어 xylose를 유일 탄소원으로 하여 성장하였다. 이 재조합 plasmid를 제한효소로 처리하여 본 결과 4.1Kb의 *Bacillus licheniformis* 유전자가 옮겨졌음을 확인했고 여기에 제한효소 HindIII와 Pvu II의 절단위치가 확인되어 제한효소 지도를 작성하였다. 이 재조합 plasmid pBGI 6는 연속계대 10일 후에도 매우 안정하게 유지되었다. 한편, 포도당 이성화 효소의 활성은 측정하여본 바 야생균주에 비해 약 20배의 증가를 나타냈다.

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